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Effect Of Antidepressants On Cytokines Profiles Seen In Inflamed Tissue And Serum Samples In Patients With Moderate Chronic Periodontitis

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EFFECT OF ANTIDEPRESSANTS ON CYTOKINES PROFILES SEEN IN INFLAMED TISSUE AND SERUM SAMPLES IN PATIENTS WITH MODERATE CHRONIC PERIODONTITIS

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EFFECT OF ANTIDEPRESSANTS ON CYTOKINES PROFILES SEEN IN INFLAMED TISSUE AND SERUM SAMPLES IN PATIENTS WITH MODERATE CHRONIC PERIODONTITIS

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ABSTRACT

This study evaluated whether depression with or without the use of antidepressant was associated with changes in inflammatory markers, intraorally and systemically, GCF volume, serum biomarker levels. Cross-sectional data were collected from individuals with moderate periodontitis who were assigned to one of three groups based upon their medical history, medication usage, and answers to a validated survey instrument that assessed their level of clinical depression. Three groups of patients were compared: 1) patients with clinically diagnosed depression taking antidepressant medications [selective serotonin reuptake inhibitors (SSRIs), norepinephrine-dopamine reuptake inhibitor (NDRIs), or serotonin and norepinephrine reuptake inhibitors (SNRIs)]. 2) patients with clinically diagnosed depression not taking any antidepressant medications 3) patients not diagnosed with depression and not taking any antidepressants. In addition, gingival tissue biopsies were collected from one periodontally affected/inflamed and one healthy/non-inflamed site in the same patient and blood samples were collected on the day of tissue biopsy for gene expression and serum biomarker assay. Tissue samples were analyzed for gene expression of inflammatory biomarkers associated with periodontal disease (IL-1β, TNF-α, IFN-γ, IL-6, IL-2, IL-8, IL-10). GCF and serum samples were analyzed for proinflammatory biomarkers (IL-1β, TNF-α, IFN-γ, IL-6, IL-2, IL-8, IL-10). This study aims to evaluate whether antidepressant use improves clinical and biological markers of disease for patients with periodontitis and depression. The results show inflammatory markers being lower in Group 1

(patients taking antidepressants) compared to the patients in Group 2 (untreated clinical depression). Anti-Inflammatory marker elevation was observed on group 1 (treated clinical depression). Inflammatory biomarkers of the group 1 (using antidepressants) were lower than the ones compared to group 3 (patients with no depression diagnosed).

Keywords: gingival tissue biopsies, GCF, serum samples, inflammatory biomarkers, periodontitis and depression, antidepressants.

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LIST OF ABBREVIATIONS

ADAA	Anxiety and Depression Association of America
cDNA	complementary deoxyribonucleic acid
GCF	gingival crevicular fluid
IFN-γ	interferon γ
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
LPS	lipopolysaccharide
MMP	matrix metalloproteinase
MDD	major depression disorder
NDRIs	norepinephrine and dopamine reuptake inhibitors
PDL	periodontal ligament
PGE2	prostaglandin E2
RT-PCR	reverse transcription polymerase chain reaction
RNA	ribonucleic acid
SSRIs	selective serotonin reuptake inhibitors
SNRIs	serotonin and norepinephrine reuptake inhibitors

TNF-α	tumor necrosis factor- alfa
UAB	University of Alabama at Birmingham
WB	western blot
kDa	kilodalton

INTRODUCTION

Across the world, mental health disorders are highly prevalent[1] with 322 million people worldwide living with depression, based on reports by Anxiety and Depression Association of America (ADAA)[2]. Despite this, mental health diseases are still significantly under-reported and improper use of medication is common[34]. An estimated 17.3 million adults in the United States have had at least one major depressive episode in the year 2017 [5], which represents 7.1% of all U.S. adults. Out of this population, 63.8% experienced severe impairment[6].

Typical treatment of mental health disorders include treatment by a health professional such as therapy, counselling and use of prescribed medications. Approximately 35% of adults with a major depressive episode did not receive treatment[6], indicating considerable underdiagnosis of mental health disorders.

Additionally, potential impact of depression and medication has been indicated in some animal models. Animal research conducted in laboratory rats demonstrated improvements in systemic inflammatory markers when rats were exposed to antidepressant medications[7,8]. Antidepressant agents have anti-inflammatory functions that may have benefits when used as adjuvants in periodontal therapy[5] but this has not been adequately researched in human subjects. In this work we will aim to observe the connection between antidepressants, periodontal disease and systemic inflammation. If depression deteriorates periodontal disease status[9,10] and patients can treat their depression effectively with antidepressants, then periodontal disease status could improve with

antidepressants and less inflammation would be observed in patients with periodontal disease taking antidepressants compared to patients not taking antidepressants.

BACKGROUND

Depression as a mental health diagnosis

Depression has been associated with periodontal disease[2]; however, its relationship to periodontal treatment outcomes has not yet been thoroughly investigated. Depression affects the hypothalamic-pituitary-adrenal axis resulting in cortisol and adrenal disturbances, immune dysfunctions, and excessive secretion of pro-inflammatory cytokines, which could potentiate immune inflammatory-mediated periodontal tissue breakdown[3,11]. Periodontal disease progression is initiated by bacterial pathogens and their byproducts inducing a host response with the production of pro-inflammatory cytokines[11]. A recent study[11] concluded that cytokine levels are altered in depressed subjects. These cytokine levels are associated with symptoms and dimensions of depression[12]. Serum cytokine levels improve with antidepressant drug administration, and these levels are related to treatment outcome of antidepressant therapies[11].

Depression association with periodontic disease

In their 2006, Johannsen et al. described increased dental plaque accumulation, gingival inflammation and elevated gene expression levels of interleukin-6 (IL-6) and cortisol in gingival crevicular fluid (GCF) in women with depression[13]. Depression has also been associated with periodontal disease in multiple investigations[12,13]. Additionally, pro-inflammatory cytokines play a role in the progression of periodontal diseases[14] and depression increases serum levels pro-inflammatory cytokines[15]. In animal models, SSRI and tianeptine (atypical antidepressant) administration alter the lipopolysaccharide (LPS)-induced cytokine profile, leading to an increase in the anti-inflammatory cytokines and a decrease in the pro-inflammatory cytokines[16].

Periodontal disease can lead to halitosis, compromised esthetics due to tooth loss which may increase psychosocial effects like isolation, shame, diminished well-being, poor self-esteem, and embarrassment. All of which have the potential to worsen clinical depression symptoms.

While many pharmacotherapies are available for the treatment of anxiety and depression, only 36.9% of those suffering receive treatment (Anxiety and Depression Association of America, ADAA, Depression, 2019). Research has shown that a roadblock to receiving medication for these mental disorders may be fear of social stigmatization[6]. Apart from the psychological impact, research has also shown that many antidepressants have side effects with oral implications, including xerostomia, which could further negatively impact periodontal treatment outcomes[2]. Conversely, patients that successfully control

their depression with medication demonstrate improved dental hygiene and a healthier lifestyle, which could potentially outweigh the immediate biochemical drawbacks[1].

This work aims to evaluate significant differences in periodontal clinical, microbiological, and immuno-inflammatory markers based on depression and medication presence. Levels of several inflammatory biomarkers in the blood such as acute phase proteins (C-reactive protein and haptoglobin) [17]; prostaglandin E2 (PGE2)[18]; secretory interlukin-1 receptor antagonist (sIL-1RA)[19–21], tumor necrosis factor- α (TNF-α) [11,16,17], and complement factors[11] have been shown to be elevated in patients with major depression disorder (MDD)[11,20]. These elevated levels of pro-inflammatory biomarkers may affect local periodontal disease progression and we postulate that effective treatment for depression may improve periodontal parameters[14]. Antidepressant agents have anti-inflammatory functions that may have benefits when used as adjuvants in periodontal therapy[5] but this has not been adequately researched in human subjects.

Antidepressants

Several categories of antidepressants are in use today. The first category of drugs eligible for this study are selective serotonin reuptake inhibitors (SSRIs), which are often the first prescribing medication[22]. These medications are safer and generally cause fewer negative side effects than other types of antidepressants. SSRIs include fluoxetine (Prozac, Selfemra), paroxetine (Paxil, Pexeva), sertraline (Zoloft), citalopram (Celexa) and escitalopram (Lexapro). SSRIs ease depression by increasing levels of serotonin in the

brain. Serotonin is one of the chemical messengers (neurotransmitters) that carry signals between brain cells. SSRIs block the reabsorption (reuptake) of serotonin in the brain, making more serotonin available[23].

The second category are serotonin and norepinephrine reuptake inhibitors (SNRIs). Examples of SNRI medications include duloxetine (Cymbalta), venlafaxine (Effexor XR), desvenlafaxine (Pristiq, Khedezla) and levomilnacipran (Fetzima). Depression is associated with reduced levels of the monoamines in the brain, such as 5-HT. The selective 5-HT and noradrenaline re-uptake inhibitors (SNRIs) are thought to restore the levels of 5-HT and noradrenaline in the synaptic cleft by binding at their re-uptake transporters preventing the re-uptake and subsequent degradation of 5-HT and noradrenaline. This re-uptake blockade leads to the accumulation of monoamines in the synaptic cleft and the concentration returns to within the normal range[24].

The third category includes norepinephrine and dopamine reuptake inhibitors (NDRIs). Bupropion (Wellbutrin, Aplenzin, Forfivo XL) falls into this category which enhance the noradrenergic transmission[25,26].

Lastly the tricyclic antidepressants are imipramine (Tofranil), nortriptyline (Pamelor), amitriptyline, doxepin, trimipramine (Surmontil), desipramine (Norpramin) and protriptyline (Vivactil) as these tend to cause more side effects than newer antidepressants, therefore they generally are not prescribed unless the patient was initially given an SSRI and no improvement has been shown. They have prevalent noradrenergic effect compared to other antidepressants[26].

Periodontal biomarkers

The ‘Biomarkers Definitions Working Group’ defines a biomarker as: ‘A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [27]. The "markers of disease" can be indicators of current disease activity, predictors of future disease progression or even predictors of future disease initiation at currently healthy sites [28]. The majority of the studies are designed to be cross-sectional, in which they compare the levels of cytokines in groups of healthy patients and patients with periodontal disease. This gives us information about the associations of biomarkers with the presence of disease and therefore test whether or not a particular marker is discriminatory[29–32].

Gingival Crevicular Fluid

Gingival crevicular fluid is a complex mixture of substances including serum, white blood cells, proteins and bacteria. Some of its functions are cleansing material from the sulcus and containment of plasma proteins that may improve adhesion of the epithelium to the tooth. They also possess antimicrobial properties. Exert antibody activity in defense of the gingiva[31,33].

GCF is formed at the rate of 0.5- 2.4 ml/day. The formation of GCF, is described with the initial fluid produced that represents interstitial fluid, which appears in the crevice because of an osmotic gradient[34,35]. This initial, pre-inflammatory fluid was considered a transudate and on stimulation, this changed to become an inflammatory exudate. In the Pashley model, the GCF production is governed by passage of interstitial fluid from capillaries tissues lymphatic system, when capillary filtrate exceeds that of lymphatic uptake, fluid will accumulate as edema and/or leave the area as GCF.

Inflammation of the gingiva, usually referred to as gingivitis, arises in the region of the crevicular epithelium well before any inflammation is clinically visible. This is sometimes referred to as clinically invisible gingivitis. As the severity of inflammation rises so does the level of GCF[31]. In most cases, the inflammation spreads to the marginal gingiva and the gingivitis becomes clinically evident.

Gingival inflammation can now be measured electronically and as a result, assessed more accurately. Because of the high correlation between gingival inflammation and GCF flow[32], GCF has been accepted as a good surrogate marker. This is further supported by its high observer-independence and quantitative nature.

Quantification of GCF

Methods of Collection of GCF Methods of Collection

Brill and Krase (1958) introduced filter paper into gingival sulcus of dogs previously injected with fluorescein within 3 min the fluorescent material was recovered on the paper strip[36]. This indicates the presence of fluid in gingival sulcus[37]. Today these measurements are commonly done with a "Periotron"[30]; an instrument designed to quantify submicrolitre volumes of fluid sampled on a filter paper strip. The Periotron measures the effect on the electrical current flow of the wetted paper tips (PerioPaper Strips comes in a sterile packet and absorb 0 - 1.2 µl of fluid). This paper strip is placed between the upper and lower jaws and the jaws are closed with a wet paper strip so the electricity flows and the machine can measure the units with energy stored in the capacitor. The Periotron measures the change in capacitance of the jaws between a dry paper strip, and a wetted one, and can provide a volume of fluid present based on that measurement[30].

Variability of GCF

There are other factors one must take into consideration in the variability of GCF. One of them is the circadian periodicity: the average flow was greater in the evening and minimal early in the morning[38]. Although, other studies show that there was no

significant differences between the flow of fluid measured at 9 a.m. and that of the fluid collected at 3 p.m[38].

It was also described by Mcluaghlin WS et al. 1993 that smoking produces an immediate but transient increase in GCF flow[39].

Presence of drugs in GCF

Drugs that are excreted through the gingival fluid may be used advantageously in periodontal therapy. Bader and Goldhaber demonstrated that intravenously administered tetracycline in dogs rapidly emerges within the sulcus[40]. Stephen et al (1980) measured the concentration of ampicillin, cephalexin, tetracycline, erythromycin, clindamycin and rifampicin in serum, saliva and GCF after a single dose administration[41]. Except on one occasion, individual GCF antibiotic concentration were equal to or considerably greater than those found in saliva.

Cytokines and periodontal disease

Cytokines have vital roles in the development and homeostasis of numerous cell types. Some of these roles are resolution of inflammation, wound healing, repair and regeneration. The periodontal disease progression is based on the interactions of these cytokines. It is now widely accepted that although the initiating factors in gingivitis and

periodontitis are the microbial elements of the dental plaque biofilm, the pathogenesis and concomitant tissue destruction is driven by the development of a chronic, inflammatory host immune response, the nature and extent of which are fundamental determinants of susceptibility and progression. The earliest cytokine studies in periodontal research can be dated back to the 1980s and coincide with the detailed characterization of IL-1, IL-2 and TNF-α[27].These experiments showed increased thymocyte proliferation induced by gingival crevicular fluid from inflamed sites compared with that from non-inflamed sites, which was presumed to be caused by cytokine-like activity[27].

Early studies focused on serum and gingival crevicular fluid samples for investigations of periodontitis- related cytokines. In recent years, periodontitis- related cytokines has come into focus as an alternative source of detecting periodontal disease [29–32]. Analysis of saliva, similarly to gingival crevicular fluid, gives a better representation of the local pathological changes in the mouth than analysis of serum. The gingival crevicular fluid content reflects inflammatory processes at individual disease sites or inflammatory status[31].

Gene expression

Genes encode proteins and proteins dictate cell function. Therefore, the thousands of genes expressed in a particular cell determine what that cell can do. Protein production starts at transcription (DNA to RNA) and continues with translation (RNA to protein).

The amounts and types of mRNA molecules in a cell reflect the function of that cell. RNA transcription makes an efficient control point because many proteins can be made from a single mRNA molecule. Transcript processing provides an additional level of regulation for eukaryotes, and the presence of a nucleus makes this possible. In eukaryotes, however, transcripts are modified in the nucleus before they are exported to the cytoplasm for translation. the primary transcripts synthesized by RNA polymerase contain sequences that will not be part of the mature RNA. These intervening sequences are called introns, and they are removed before the mature mRNA leaves the nucleus. The remaining regions of the transcript, which include the protein-coding regions, are called exons, and they are spliced together to produce the mature mRNA. Eukaryotic transcripts are also modified at their ends, which affects their stability and translation.

Pro-inflammatory cytokines

Cytokines are small secreted proteins released by cells which have a specific effect on the interactions and communications between cells.

Pro-inflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are involved in the process of pathological pain and inflammation [42].

Interleukin - 1

The IL-1 family of cytokines is defined based on structural similarity and comprises 11 members, divided into three subfamilies. In terms of pathogenesis, the IL-1 cytokines are generally pro-inflammatory, but interleukin-1 receptor antagonist and interleukin-36 receptor antagonist have antagonistic functions. Interleukin-1beta is the prototypical interleukin-1.

IL-1β is released primarily by monocytes and macrophages as well as by non-immune cells, such as fibroblasts and endothelial cells, during cell injury, infection, invasion, and inflammation.

IL-1β in the western blot assay, IL1- beta has an observable band size of 35 kDa.

In periodontitis, IL-1β is associated with neutrophil recruitment and activation of osteoclasts through its ability to induce chemokines and activate osteoclasts. In case control studies, nine of the 13 show a significantly higher IL-1β level in patients with periodontitis compared with healthy controls.

Tumor Necrosis Factor-Alpha

TNF-α is another inflammatory cytokine that plays a well-established role in the inflammatory process. It is involved in cell proliferation, apoptosis and morphogenesis, as well as in host immune defense. TNF- α is produced mainly by macrophages as a primary response of toll-like receptor signaling, by activated T-cells and natural killer

cells, and also by nonimmune cells, including endothelial cells and fibroblasts. Both TNF- α and RANKL belong to the tumor necrosis factor superfamily, and both are potent activators of osteoclasts and thereby mediate bone resorption. TNF- α has an observable band size of 26 kDa and additional bands at 48 kDa.

Interleukin - 6

IL-6 is produced by innate immune cells such as macrophages and dendritic cells, but also by some CD4+T-cells, as well as by non-immune cells such as fibroblasts and endothelial cells. Interleukin-6 is elevated in many inflammatory diseases and mainly functions to activate B-lymphocytes in addition to having a role in influencing the balance of CD4+ effector T-cell populations and potentially influencing myeloid cell differentiation. The predicted molecular weight of IL-6 is of 17,25 kDa. The band observed at 50 kDa may represent multimers of IL6 as reported in the literature.

Interferon - gamma

IFN-γ has a predicted molecular weight that can be observed at 25 kDa. It is a cytokine that is critical for innate and adaptive immunity against viral, bacterial and protozoal infections. It is an activator of macrophages. Abnormal IFN-γ expression is associated with autoinflammatory and autoimmune diseases. IFN-γ is produced in mainly by natural killer (NK) cells.

Anti-inflammatory cytokines

The anti-inflammatory cytokines are a series of immunoregulatory molecules that control the pro-inflammatory cytokine response. Cytokines act in concert with specific cytokine inhibitors and soluble cytokine receptors to regulate the human immune response. Their physiologic role in inflammation and pathologic role in systemic inflammatory states are increasingly recognized. Among all the anti-inflammatory cytokines, IL-10 is a cytokine with potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1 by activated macrophages. In addition, IL-10 can up-regulate endogenous anti-cytokines and down-regulate pro-inflammatory cytokine receptors. Thus, it can counter-regulate production and function of pro-inflammatory cytokines at multiple levels.

Interleukin - 10

Interleukin-10 is widely produced by innate and adaptive immune cells alike and is a key anti-inflammatory cytokine, involved in inhibiting and regulating proinflammatory immune responses and in promoting resolution of inflammation.

Interleukin - 8

IL-8 has a predicted molecular weight of 8-12 kDa. It is produced by macrophages and epithelial cells. IL-8 is stored in endothelial cells and it is known as a neutrophil chemotactic factor. It has two primary functions: it induces chemotaxis in target cells, primarily neutrophils but also in other granulocytes and this causes them to migrate toward the site of infection Il-8 also stimulates phagocytosis once they have arrived and it is a potent promoter of angiogenesis.

Interleukin - 2

IL-2 is a signaling molecule in the immune system it is a 15kDa protein that regulates leucocytes. It is part of the natural response to microbial infection. The major source of IL-2 are activated CD4+Tcells, activated CD8+ T cells, Natural killer cells, dendritic cells and macrophages.

Cyclooxygenase-2

COX-2 is one of the two cyclooxygenases, it is involved in the conversion of arachidonic acid to prostaglandin H2, which is an important precursor of prostacyclin, which is expressed in inflammation.

1 Cytokines observed in this study

Cytokines	**Principal Source**	**Primary Activity**
IL-6	Activated Th2 cells, APCs, other somatic cells	Acute phase response, B cell proliferation, thrombopoiesis, synergistic with IL-1 and TNF on T cells
IL-8	macrophages, somatic cells	Chemoattractant for neutrophils and T cells
IL-10	Activated Th2 cells, CD8+ T and B cells, macrophages	Inhibits cytokine production, promotes B cell proliferation and antibody production, suppresses cellular immunity, mast cell growth
IFN-γ	Activated Th1 and NK cells	Induces of class I MHC on all somatic cells, induces class II MHC on APCs and somatic cells, activates macrophages, neutrophils, NK cells, promotes cell-mediated immunity, antiviral effects
TNF-α	macrophages, mast cells, NK cells, sensory neurons	Cell death, inflammation, pain
IL1-β	Macrophages and another antigen presenting cells (APCs)	Costimulation of APCs and T cells, inflammation and fever, acute phase response, hematopoiesis

OBJECTIVES

The primary objective in this study is to evaluate whether the use of antidepressants can reduce the amount of inflammation as measured by inflammatory markers. The secondary goal of the study is to evaluate whether there is an observable systemic response that correlates with the local inflammatory markers. This work seeks to elucidate if elevated inflammatory markers are associated with untreated clinical depression in patients with periodontitis when compared to those without depression and individuals with treated depression. Increased inflammatory marker expression in patients with untreated clinical depression could result in worsening prognosis and poor response after periodontal treatment. Further studies will be needed in order to confirm the effect of clinical depression during and after active periodontal treatment. In addition, this work validates the use of the Personal Health Questionnaire Depression Scale (PHQ8) survey instrument as a screening tool for clinical depression in a dental setting. This may be a useful tool in interdisciplinary management of significant mental health conditions in patients seeking dental care.

The demographics information that was gathered was: Age, Gender, BMI and Race.

MATERIALS AND METHODS

This was an interdisciplinary project in which 550 patients were screened and from these, 92 patients with chronic periodontitis were enrolled into the study. The department of psychiatric research provided us with the Personal Health Questionnaire Depression Scale (PHQ8), an established tool to evaluate presence of depression. Based on the score of the PHQ8 questionnaire and the psychiatric history of preexisting clinical depression status or lack of it given by the psychiatrist, the patients were placed into one of the 3 groups listed below:

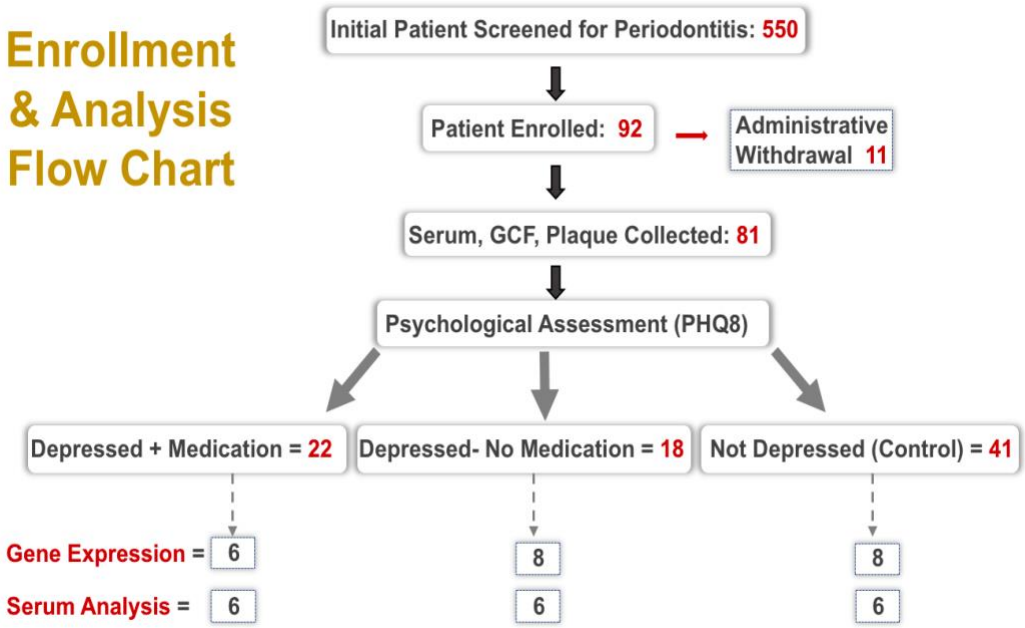
- Group 1: Twenty-two patients with clinically diagnosed depression taking medication (SSRIs, NDRIs, SNRIs)
- Group 2: Eighteen patients with clinically diagnosed depression not taking any antidepressants (SSRIs NDRIs, SNRIs and Tricyclic antidepressants).
- Group 3: Forty-one patients without depression, not taking any antidepressants.
-

Screening process

The study was conducted using facilities in the UAB School of Dentistry Building as well as the password and firewall protected computers issued through UAB School of Dentistry for work and patient-related purposes.

This was a single site study at UAB with only one same person collecting the samples and doing the questionnaires.

It was the responsibility of Dr. Sarah Startley to keep the identity of the patients protected and well organized.



1 Enrollment and analysis flow chart.

After the patients were divided by group using the PHQ8, 6-8 different patients' sample were used for the following analysis. All 81-patient had Serum GCF and plaque samples collected. 3 samples in different sites for GCF and plaque. And 1 vial 10 ml of serum sample were collected for all 81 patients. Two different tissue samples, a healthy and an affected tissue, were collected for 22 patients giving us a total of 44 samples to analyze.

Inclusion and exclusion criteria

2 Inclusion and exclusion criteria for enrollment in study

Inclusion Criteria	Exclusion Criteria
English speaking	Non-English speaking
At least 18 years old	Less than 18 years old
Must be a patient of the UAB Dental School	Smokers/tobacco users (>10 cigarettes/day)
Able to read and understand informed consent document	Any dental condition that requires immediate treatment, such as emergency care
Good general health as evidenced by medical history.	Chronic use (≥3 times/week) of anti-inflammatory medications (e.g., non-steroidal anti-inflammatory drugs, steroids). Low-dose aspirin (less than 325 mg daily).
Minimum of 18 teeth, excluding third molars	Immunocompromised subjects
Having moderate periodontal disease according to the NIH definition (at least 2 teeth PD and ≥4 mm CAL at baseline) .[1]	Any medical history or any concomitant medication that might affect the assessment of the study treatment or periodontal tissues, such as uncontrolled diabetes, nifedipine, phenytoin (Dilantin), subjects with arthritis and morbidly obese (BMI ≥ 40)
Having >30 percent bleeding sites upon probing	Patients with cleanings less than 3 months to the collection of samples.

[1] Using the cdc-aap definition of moderate periodontitis: moderate periodontal disease is defined as having at least two teeth with interproximal attachment loss of 4 millimeters or more or at least two teeth with 5 millimeters or more of pocket depth at interproximal sites.

Patients taking depression medication with chronic periodontitis	
Patients without depression with chronic periodontitis	

Patient population

A total of 92 patients were consented to the study. 11 patients were excluded from the study due to not meeting the criteria after enrollment. The remaining 81 patients underwent periodontal clinical status evaluation before periodontal treatment. Clinical examinations recorded periodontal probing depth, clinical attachment level, plaque index, and bleeding index. All of the patients exhibited moderate chronic periodontal disease.

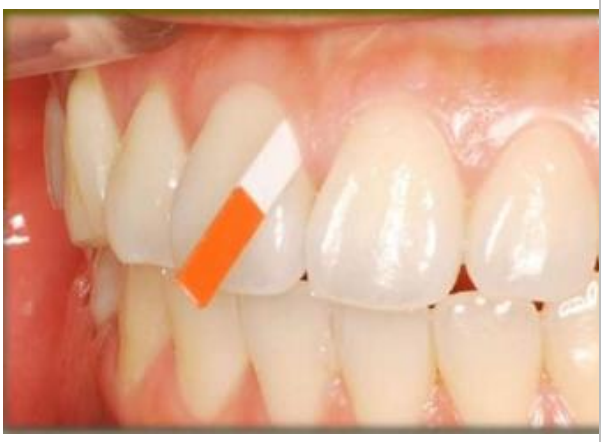
3 Description of groups and number of patients enrolled.

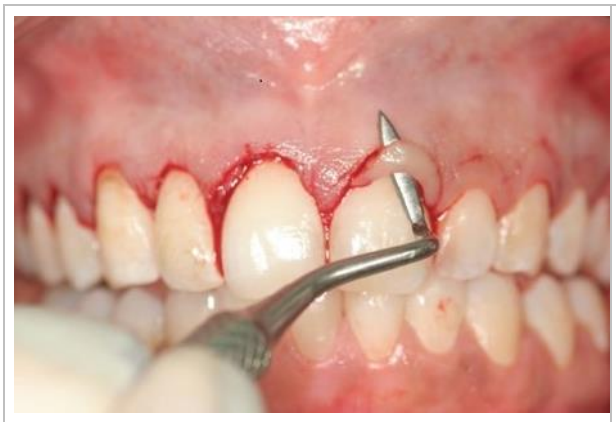
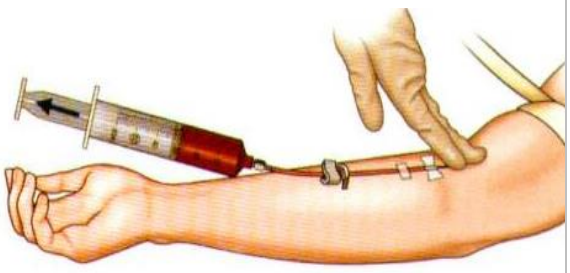
Group	**Treatment**	**Number of participants**
Group 1 Depressed with medication	Patients with clinically diagnosed depression taking (SSRIs, NDRIs, SNRIs, tricyclic antidepressants)	22
Group 2 Depressed	Patients with clinically diagnosed depression not taking any antidepressants (SSRIs NDRIs, SNRIs and Tricyclic antidepressants)	18
Group 3 Control	Patients without depression	41

Outcome measurement

The outcomes were measured in the following way. The PHQ8 questionnaire was measuring the depression status. As for the clinical part the gingival crevicular fluid (GCF) was measuring oral inflammatory markers at a local level. This was also confirmed with the tissue samples collected, since the goal was to measure gene expression in each group. The serum sample was taken to analyze the circulating cytokine levels at a systemic level and compare and contrast local and systemic inflammatory gene expression.

4 Samples collected.

	Collection of GCF (Gingival Crevicular Fluid): accomplished by using absorbent filter paper strips inserted into the gingival crevice or periodontal pocket for 30 seconds.
	Collection of plaque samples: accomplished by gently scrapping exposed tooth surfaces with a sterile dental scaler

	Collection of tissue sample: accomplished by removing tissue that would be discarded during periodontal surgeries.
	Collection of blood serum: Accomplished by blood draw and centrifuge

Sample collection:

Soft tissue, GCF, subgingival plaque, and blood serum were collected from each patient immediately prior to resective periodontal surgical procedures to treat periodontal disease. All samples from each individual patient were collected on the same day. Briefly, sample collection is described as follows: soft tissue biopsies were collected from both affected (sites with probing depths (PD) ≥4 mm) and healthy sites (sites with PD <4 mm). At these sites, full thickness epithelialized tissues that included the sulcular wall were harvested from patients at both affected and healthy sites during resective surgical therapy. Samples were labeled and placed in amnion solution, frozen in liquid nitrogen, and stored in -80º C freezer until batch assays were performed. GCF was

collected from the three deepest PD sites using paper strips which were placed inside the pockets for exactly 30 seconds. The quantity of GCF was immediately measured with the Periotron 8000 (Oraflow, Inc. Smithtown, NY). After thorough supragingival debridement subgingival plaque samples were collected with paper points at the two deepest PD sites and samples were pooled. Plaque samples were placed in a TE buffer solution and frozen immediately after removing them from the patient's mouth. Blood samples were withdrawn from the antecubital vein with a 18 or 22-gauge butterfly needle, collected in a serum blood collection test tube spray-coated with silica and then left to rest for 30 minutes before being centrifuged for 15 minutes at 3000 rpm to separate the serum. Serum was extracted and aliquoted into 3 mL aliquots in Eppendorf tubes. All 4 types of samples were transported in liquid nitrogen and stored in a standardized -80º C freezer.

Sample analysis:

Soft tissue gene expression analysis:

The levels of inflammatory marker gene expression were determined using polymerase chain reaction (PCR). First the tissues were directly placed in an amnion solution to preserve intact cells and allow transportation to the laboratory facility. Tissue samples were then removed from the amnion solution and placed in a standardized -80º C freezer. Batch testing of samples was performed to insure consistency of procedures and reagents.

All soft tissue samples were dissolved using Trizol agent to preserve the RNA and DNA while eliminating proteins within the tissue.

Trizol RNA Isolation Protocol:[43]

1. Tissue samples were homogenized in 0.5 ml of Trizol reagent per 50 to 75 mg of tissue. The sample volume did not exceed 10% of the volume of Trizol Reagent used.
2. Tissue was left at least 3 hours and vibrated vigorously using an ultrasound vibrator to help disintegrate the tissue in the Trizol reagent.
3. 0.1 ml of chloroform was added per 0.5 ml of Trizol reagent. Samples were vortexed vigorously for 15 seconds and incubated at room temperature for 2 to 3 minutes.
4. The samples were then centrifuged at 12,000 g for 15 minutes at 2 to 8º C. Following centrifugation, the mixture was separated into lower red, phenol-chloroform phase, interphase, and a colorless upper aqueous phase. As RNA remains exclusively in the aqueous phase, this phase was transferred without disturbing the interphase into a fresh tube.
5. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. Samples were incubated at 15 to 30º C for 10 minutes and centrifuged at 12,000 g for 10 minutes at 2 to 4º C.

6. The supernatant was then removed completely to leave the RNA pellet in the tube. The RNA pellet was then washed twice with 75% ethanol, adding at least 0.5 ml of 75% ethanol per 0.5 ml of Trizol reagent used for initial homogenization.
7. The samples were mixed by vortexing and centrifuged at 7,500 g for 5 minutes at 2-8ºC. The RNA pellet was air dried and dissolved in 20 ml of DEPC-treated water.

The nucleic acid concentrations were standardized and triplicate assays from the same soft tissue sample were run in the rt PCR machine to reduce human error.

Expression of genetic material for IL-1β, TNF-α, IFN-γ, IL-6, IL-2, IL-8, IL-10 were assessed from each sample and compared between the 3 groups via gel electrophoresis in ethidium bromide gel and compared to a standard DNA ladder for quantification.

Serum analysis

Serum samples were aliquoted into 3 mL aliquots and flash frozen in Eppendorf tubes immediately after harvest using liquid nitrogen. They were then transported to the laboratory and stored at -80º C until batch testing. Serum samples were assayed for Western Blot technique and compared against control samples of known concentration obtained from the agarose gel electrophoresis. Comparison of concentrations were accomplished through 2 cm^3 of serum.

Western Blot protocol[44]:

The purpose of western blotting is to separate proteins on a gel according to the molecular weight. The proteins are then transferred onto a membrane where they can be detected using antibodies. Heat the samples and 95º C for five to 10 minutes in a sample buffer containing a reducing agent such as beta mercaptoethanol. This results in linearized proteins with a negative charge proportional to their size.

RT-PCR Primers

RT-PCR amplification of a particular RNA sequence requires two PCR primers that are specific for the gene transcript of interest. The first step was to design the primers. The primer design should allow differentiation between the amplified product from cDNA and an amplified product derived from the sample genomic DNA.

5 Primers

Human Primers	Length	Sequence	Cycle condition (Tm)
IL-6 Forward	21	AAT TCG GTA CAT CCT CGA CGG	3 ODs = 14.8 nmol = 95.1 µgrams
IL-6 Reverse	20	GGT TGT TTT CTG CCA GTG CC	3 ODs = 17.1 nmol = 104.3 µgrams
IL-1β Forward	23	GCA GCC ATG GCA GAA GTA CCT GA	3 ODs = 13.1 nmol

Human Primers	Length	Sequence	Cycle condition (Tm)
			= 92.9 µgrams
IL-1β Reverse	22	CCA GAG GGC AGA GGT CCA GGT C	3 ODs = 13.8 nmol = 94.1 µgrams
IL-10 Forward	20	GGT TGC CAA GCC TTG TCT GA	3 ODs = 16.3 nmol = 99.6 µgrams
IL-10 Reverse	19	AGG GAG TTC ACA TGC GCC T	3 ODs = 16.5 nmol = 96.3 µgrams
IL-8 Forward	19	CTT CTC CAC AAC CCT CTG CAC	3 ODs = 17 nmol = 97 µgrams
IL-8 Reverse	21	CTT CTC CAC AAC CCT CTG CAC	3 ODs = 16.9 nmol = 104.9 µgrams
IL-2 Forward	23	AAG AAT CCC AAA CTA ACC AGG AT	3 ODs = 12.6 nmol = 88.6 µgrams
IL-2 Reverse	27	TCT AGA CAT GAA GAT GTT TCA GTT CTC	3 ODs = 11.5 nmol = 95 µgrams
TNF-α Forward	21	ATC TTC TCG AAC CCC GAG TGA	3 ODs = 15.1 nmol = 95.9 µgrams

Human Primers	Length	Sequence	Cycle condition (Tm)
TNF-α Reverse	19	CGG TTC AGC CAC TGG AGC T	3 ODs = 17.3 nmol = 100.1 µgrams
IFN-γ Forward	20	TCC CAT GGG TTG TGT GTT TA	3 ODs = 16.1 nmol = 99 µgrams
IFN-γ Reverse	20	AAG CAC CAG GCA TGA AAT CT	3 ODs = 14.9 nmol = 91.1 µgrams
COX-2 Forward	27	TTC AAA TGA GAT TGT GGG AAA ATT GCT	3 ODs = 11.1 nmol = 92.7 µgrams
COX-2 Reverse	25	AGA TCA TCT CTG CCT GAG TAT CTT T	3 ODs = 12.9 nmol = 98 µgrams
GAPDH Forward	20	GTC GCT GTT GAA GTC AGA GG	3 ODs = 15.2 nmol = 94.5 µgrams
GAPDH Reverse	18	GAA ACT GTG GCG TGA TGG	3 ODs = 16.8 nmol = 94.2 µgrams
Beta-actin Forward	19	TTG CCG ACA GGA TGC AGA A	3 ODs = 15.7 nmol = 92.3 µgrams
Beta-actin Reverse	20	GCC GAT CCA CAC GGA GTA CT	3 ODs = 15.7 nmol = 95.5 µgrams

Laboratory analysis

Internal control

The importance of having an internal control within every step is to make sure that one is doing each step correctly and that the sample is always present in every group.

Beta Actin was used as a control for RT-PCR tissue samples. Duplicates and triplicates were done for each sample to reduce variability. Albumin was used as the internal control for Western blot assay. Its predicted band size is of 69 kDa.

RT-PCR

PrimeScript RT reagent Kit was used to perform the reverse transcription optimized for real-time RT-PCR. Once the cDNA was acquired real-time PCR was possible. The real-time PCR reagent used was SYBR®Premix Ex Taq™ II (Tli RNaseH Plus). All the components were enough for 200 reactions, 10 µl per reactions.

1. 5X PrimeScript Buffer (for Real Time) *1 400 µl
2. PrimeScript RT Enzyme Mix I*2 100 µl
3. Oligo dT Primer 50 µM 100 µl
4. Random 6 mers 100 µM 400 µl
5. RNase Free dH2O 1 ml

6. EASY Dilution (for Real Time PCR)*3 1 ml

   *1 Contains dNTP Mixture and Mg2+.

   *2 Contains RNase Inhibitor.

   *3 To be used when producing serial dilutions of total RNA and cDNA.

EASY Dilution (for Real Time PCR) makes it possible to obtain a precise dilution down to very low concentrations. One must be careful when using since compatibility with products from other manufacturers has not been verified.

Agarose electrophoresis

One of the most effective ways of separating DNA fragments is with agarose gel electrophoresis. Agarose 2% is casted by gelation and its' polymers associate non-covalently and form a network of bundles whose pore sizes determine a gel's molecular filtering properties. To separate DNA using agarose gel electrophoresis, the DNA is loaded into pre-cast wells in the gel and a current applied. The phosphate backbone of the DNA (and RNA) molecule is negatively charged, so when it is placed in an electric field, DNA fragments will migrate to the positively charged anode. DNA will separate according to its molecular weight. The rate of migration can be determined by the size of DNA molecule, the agarose concentration, the DNA conformation, the voltage applied, presence of ethidium bromide, type of agarose and the electrophoresis buffer. After separation, the DNA molecules can be visualized under uv light after staining with an appropriate dye[45].

Western blot

This protocol was used to separate the proteins on a gel according to the molecular weight. The proteins are then transferred onto a membrane where they were detected using 5 specific antibodies. TNF-α, IFN-γ , IL1-β, IL-8 and IL-6.

The samples were heated in a 95° C for five to 10 minutes in a sample buffer containing a reducing agent, beta mercaptoethanol. this results in linearized proteins with a negative charge proportional to their size.

Before doing the western blotting, specific antibodies were carefully selected with previous agarose electrophoresis to identify the proteins that have been separated based on size.

Western Blot Protocol:

The gel was placed into the electrophoresis thank and the buffer was added, ensuring that all the wells are covered. The molecular marker was placed in into the first lane and then all of the other wells adjacent to it were loaded. All of them containing equal amounts of the sample. Once the samples are loaded the running buffer was placed. The lid was placed onto the electrophoresis thank. The voltage was set as recommended by the Abcam manufacturer of the gels in the tank. The gel was run until the die front moved sufficiently down the gel. In some cases, it was run all the way till the end of the gel and

in other cases not all the way to the end, this was depending on the molecular weight of the protein that we wanted to observe.

The proteins were transferred from the gel onto a membrane. This membrane was made out of PVDF. The gel was removed from the tank and carefully released from the plastic case. The wells were cut up and placed into the transfer buffer.

Sample preparation for Western blot

Sample preparation from serum:

The serum sample of each patient was placed on ice and worked with as quickly as possible to prevent degradation by proteases. 500ul of serum sample to 50 ul of RIPA buffer (radioimmunoprecipitation assay buffer). Then the samples were reduced and denatured, by boiling each cell lysate in sample buffer at 100°C for 4-5 min. Lysates can be aliquoted and stored at -20°C for future use. Centrifuge for 18-20 min at 12,000 rpm at 4°C in a microcentrifuge. Gently remove the tubes from the centrifuge and place on ice, transfer the supernatant into a fresh tube kept on ice and discard the rest.

Loading and running the gel

Load equal amounts of protein into the wells of the SDS gel, along with molecular weight marker. 50 μg of total protein from serum sample were loaded in each well.

Run the gel for 1–2 h at 100 V. This will transfer the protein from the gel to the membrane.

The membrane can be either nitrocellulose or PVDF. In this case PVDF was used.

PVDF membrane has to be activated with methanol for 1 min and rinse with transfer buffer before preparing the stack. The time and voltage of transfer may require some optimization. It was run to transfer for 45-50 min at 100 V.

Transfer of proteins to the membrane can be checked using Ponceau S staining before the blocking step.

Antibody staining.

The membrane was blocked for 1 hour at room temperature blocking buffer, 5% milk. Then it was incubated with appropriate dilutions of primary antibody in blocking buffer. Overnight incubation at 4°C in order to optimize. The membrane then was washed in three washes of PBST, 5 min each with PBST. Then the secondary antibody was added to the membrane and incubated for 1 hour at room temperature. It was washed in three washes of PBST, 5 min each.

And stored at 4 degrees in a dark container until scanned and analyzed.

Stripping and re-staining

Membranes can be stripped of antibodies for use in subsequent rounds of Western detection. This allows to reuse the same membrane for investigation of different proteins and saves both time and material. Blots can be stripped and reproved several times, but

each round of stripping removes some sample from the blot. This decreases the sensitivity of later rounds of detection and this means that they need longer exposure times or more sensitive methods.

If detecting proteins of different weights or when using different antibodies with different binding affinities, first detect the protein with the lower expected signal sensitivity. It is better to use PVDF membranes since they are more durable and resist loss of sample better than nitrocellulose membranes.

RESULTS

Statistical analysis

The demographics information that was gathered was: Age, Gender, BMI and Race.

The measurements that were studied in a clinical level were the GCF and Gene expression of IFN-γ, TNF-α, IL1-β, IL-6, IL-8 IL-10 Il-2 and COX-2.

GCF was taken from 3 different sites for each patient and the average of these 3 measurements was used to compare the three different groups. This is the tool for us to compare the statistical value of objective measurements such as Cytokines and amount of GCF.

For the rtPCR the gene expression of in Tissue samples: IFN-γ, TNF-α, IL1-β, IL-6, IL-8 IL-10 Il-2 and COX-2 were analyzed to observe inflammatory cytokines locally.

For the Western blot the gene expression of in serum samples: IFN-γ, TNF-α, IL1-β, IL-6, IL-8 were analyzed to observe inflammatory cytokines systemically.

Demographics characteristics between three groups

The demographics are important to understand the population and its distribution. It also help us to observe if the distribution was equal within the groups. According to the tables below it seems as if the samples were evenly distributed within each group.

6 Race and gender of population in the study.

ALL (n=81)	Frequency	Percent	Cumulative Frequency	Cumulative Percent
Race				
Caucasian	55	67.90	55	67.90
African American	17	20.99	72	88.89
Others	9	11.11	81	100.00
Gender				
Female	40	49.38	40	49.38
Male	41	50.62	81	100.00

7 Age and BMI of population in this study.

ALL (n=81)	Mean	Std Dev	Median	Inter-Quartile Range
Age	64.5	11.8	65.0	11.0
BMI	27.5	5.0	26.7	6.9

8 Race and gender of population in the study by group

ALL (n=81)	Group 1 (n=22)	Group 2 (n=18)	Group 3 (n=41)
Race			
Caucasian	15 (68.18%)	13 (72.22%)	27 (65.85%)
African American	4 (18.18%)	3 (16.67%)	10 (24.39%)
Other	3 (13.64%)	2 (11.11%)	4 (9.76%)
Gender			
Female	13 (59%)	12 (66.7%)	15 (36.6%)
Male	9 (40%)	6 (33.3%)	26 (63.4%)

9 Age and BMI of population in this study by group.

ALL (n=81)	AGE		BMI	
	Mean	Std Dev	Mean	Std Dev
Group 1 (n=22)	65.5	10.4	27.0	5.0
Group 2 (n=18)	61.9	11.5	28.8	5.9
Group 3 (n=41)	65.0	12.7	27.3	4.7

Gingival crevicular fluid

10 Average GCF of population in the study by group

ALL (n=81)	GCF	
	Mean	Std Dev
Group 1 (n=22)	**75.7**	29.3
Group 2 (n=18)	148.9	22.3
Group 3 (n=41)	133.8	29.07

11 Kruskal-Wallis Test for GCF average

Chi-square	38.2122
DF	2
Pr> Chi-Square	<.0001

12 Average GCF of population in the study compared within groups

Group	Wilcoxon Z	DSCF Value	Pr > DSCF
1 vs. 2	-5.0307	7.1144	**<.0001**
1 vs.3	-5.3853	7.6159	**<.0001**
2 vs. 3	2.1485	3.0384	0.0803

Tissue Analysis- Gene expression

13 Mean Affected tissue gene expression determined with rt-PCR by group

Gene	Group 1	Group 2	Group 3	P value	Group 1 vs. Group 2 (P value)	Group 2 vs. Group 3 (P value)	Group 1 vs. Group 3 (P value)
IFN-γ	$3.27x10^{-3}$	$20.1x10^{-3}$	$6.73x10^{-3}$	**0.0357**	**0.0545**	0.7300	0.0942
TNF-α	$0.65x10^{-3}$	$0.61x10^{-3}$	$0.30x10^{-3}$	0.0749	0.9675	0.4425	**0.0373**
IL1-β	$5.93x10^{-3}$	$6.89x10^{-3}$	$0.30x10^{-3}$	0.7414	0.7593	0.9334	0.8322
IL-6	$16.4x10^{-3}$	$48.7x10^{-3}$	$4.05x10^{-3}$	0.0580	0.2451	0.4059	0.0936
IL-8	$9.78x10^{-3}$	$4.73x10^{-3}$	$34.4x10^{-3}$	**0.0275**	0.5494	0.1805	**0.0380**
IL-10	$4.36x10^{-3}$	$3.11x10^{-3}$	$14.2x10^{-3}$	0.3966	1.0000	0.4385	0.5561
IL-2	$77.9x10^{3}$	$0.36x10^{3}$	$0.01x10^{-3}$	0.6703	0.8956	0.5768	0.8956
COX-2	$0.38x10^{-3}$	$0.23x10^{-3}$	$0.25x10^{-3}$	0.6054	0.5386	0.8956	0.8761

14 Mean Healthy tissue gene expression determined with rt-PCR by group

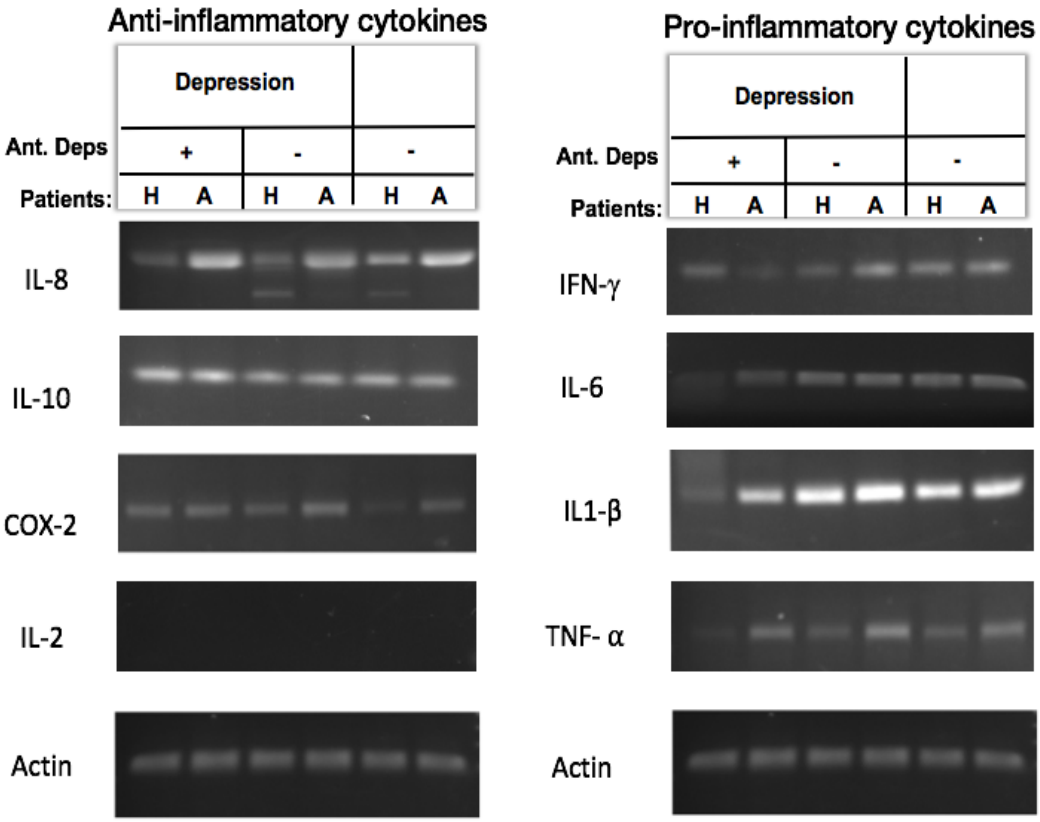
Gene	Group 1	Group 2	Group 3	P value	Group 1 vs. Group 2 (P value)	Group 2 vs. Group 3 (P value)	Group 1 vs. Group 3 (P value)
IFN-γ	$2.00x10^{-3}$	$2.21x10^{-3}$	$2.87x10^{-3}$	0.6570	0.8956	0.8633	0.6321
TNF-α	$0.42x10^{-3}$	$0.72x10^{-3}$	$0.33x10^{-3}$	0.1300	0.3186	0.9047	0.1333
IL1-β	$1.85x10^{-3}$	$4.11x10^{-3}$	$0.79x10^{-3}$	**0.0116**	**0.0498**	0.3335	**0.0526**
IL-6	$3.43x10^{-3}$	$46.2x10^{-3}$	$37.2x10^{-3}$	0.8373	0.8354	0.9888	0.8956
IL-8	$4.17x10^{-3}$	$3.91x10^{-3}$	$3.99x10^{-3}$	0.8760	0.9473	0.8476	1.0000
IL-10	$10.2x10^{-3}$	$3.6x10^{-3}$	$8.01x10^{-3}$	0.5031	0.6321	0.9860	0.5169

IL-2	$0.52x10^{3}$	$443x10^{3}$	$207x10^{3}$	0.7209	1.0000	0.5897	1.0000
COX-2	$0.89x10^{-3}$	$0.52x10^{-3}$	$0.19x10^{-3}$	0.6646	0.8956	0.9818	0.5561

Comparing expression between healthy and affected for each group

15 Mean Affected vs Healthy tissue gene expression determined with rt-PCR by group

Gene	Group 1	Group 2	Group 3	Group 1 (P value)	Group 2 (P value)	Group 3 (P value)
IFN-γ	$-0.01x10^{-3}$	$9.32x10^{-3}$	$1.17x10^{-3}$	1.0000	1.0000	0.8438
TNF-α	$0.28x10^{-3}$	$-0.13x10^{-3}$	$-0.02x10^{-3}$	0.2500	1.0000	1.0000
IL1-β	$3.78x10^{-3}$	$4.27x10^{-3}$	$8.67x10^{-3}$	0.5000	0.2500	1.0000
IL-6	$12.9x10^{-3}$	$2.56x10^{-3}$	$2.33x10^{-3}$	**0.0313**	0.9453	**0.0313**
IL-8	$5.45x10^{-3}$	$-1.01x10^{-3}$	$32.9x10^{-3}$	0.7500	1.0000	0.2500
IL-10	$-6.9x10^{-3}$	$-1.50x10^{-3}$	$9.65x10^{-3}$	0.8750	0.6250	1.0000
IL-2	$156x10^{3}$	NA	NA	1.0000	NA	NA
COX-2	$0.33x10^{-3}$	$-0.34x10^{3}$	$0.04x10^{-3}$	0.5000	0.5000	0.2500



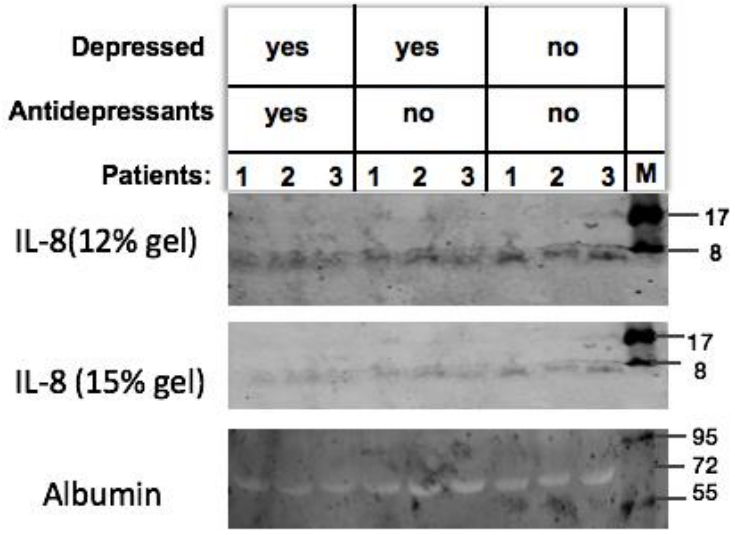
2 Anti-inflammatory and pro inflammatory cytokines expressed in agarose electrophoresis.

In the agarose electrophoresis we could observe the gene expression in the anti-inflammatory cytokines and the pro-inflammatory cytokines.

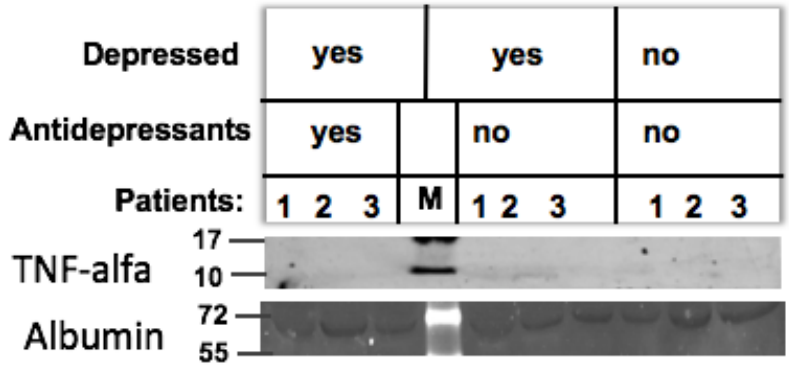
Same concentrations of cDNA were placed in each of the agarose wells, after electrophoresis we obtained these figures in which we could see if there was any expression of the genes or what was the density related to the expression of each gene. In the first two wells we can observe the patient from group 1 which was diagnosed with depression and is also taking antidepressant medication in the first column H stands for

healthy tissue obtained from this patient and the second column A stands for the affected tissue taken from this same patient. We can observe that IL 8, IL10 and COX-2 are not only present but have more density compared to the other columns. There was no gene expression for IL-2 which correlates with the results obtained from the rt PCR. Beta-Actin was the internal control and it is equally present in all the wells.

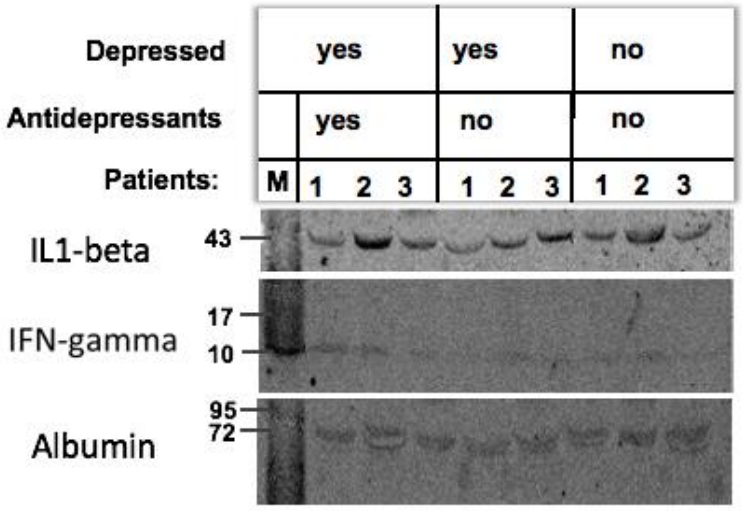
From the pro-inflammatory cytokines, we can observe that there is a very weak signal associated with the gene expression of IFN-γ, IL1-α, TNF-β and IL-6 in the first two columns which are precisely Group 1 patients, diagnosed with depression and taking antidepressant medication. We can observe the opposite behavior of gene expression related to group 2 and group 3 patients.



3 Western blot analysis of IL-8 expressed in serum samples with albumin as internal control. Marker on the left of the image.



4 Western blot analysis of TNF-α expressed in serum samples with albumin as internal control. marker on the middle of the image.

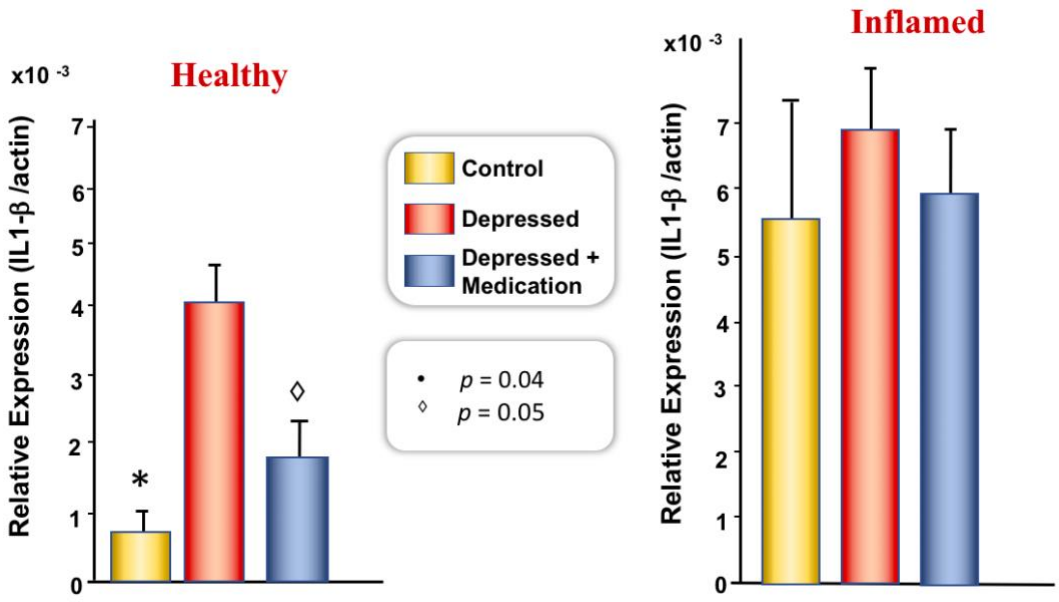


5 Western blot analysis of IL1-β and IFN-γ expressed in serum samples with albumin as internal control. Marker on the right of the image.

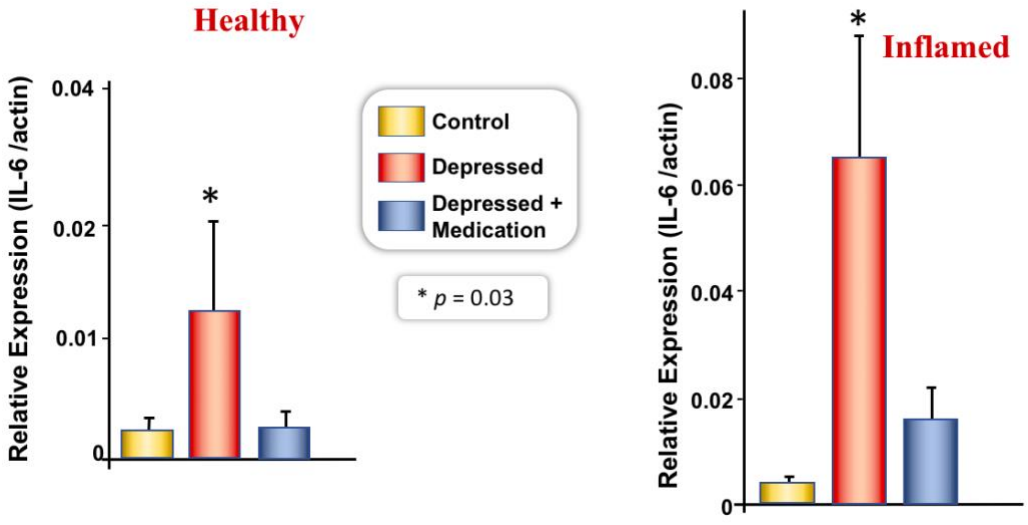
There was gene expression present in the serum samples for IL1-β and IL-8. The control internal control to show that all samples were present in the membrane was Albumin whose band size is expected to be at 69 kDa.

For the gene IL-8, two different gels were cast, first of 12% and the second time of 15%, this was to have a clear image of the presence of this gene.

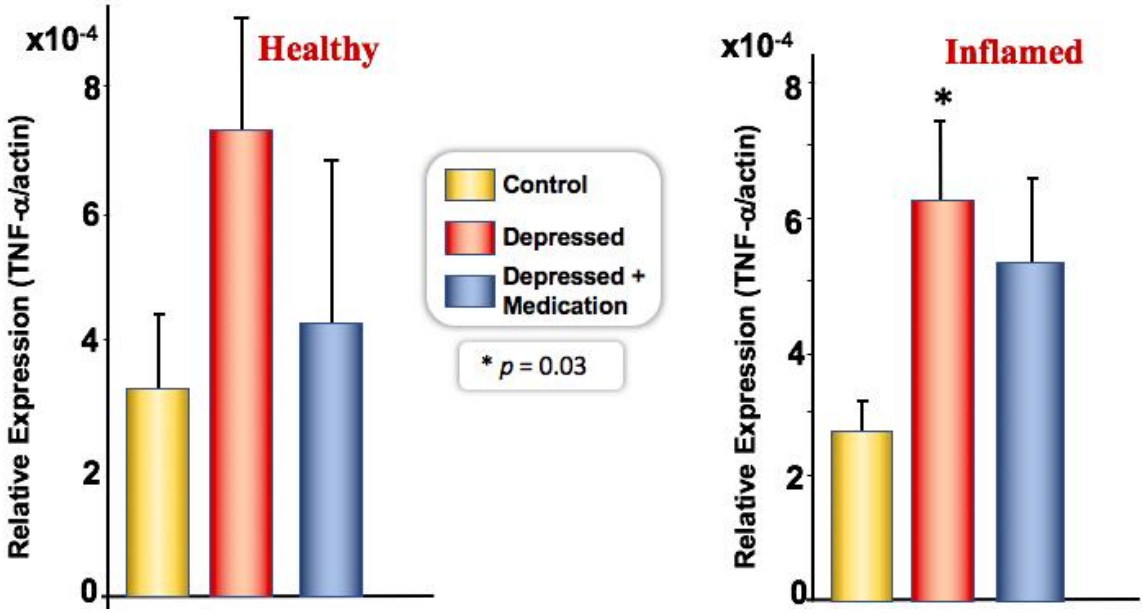
For TNF-α and IFN-γ, there was not a clear image of the band, it was barely observable.



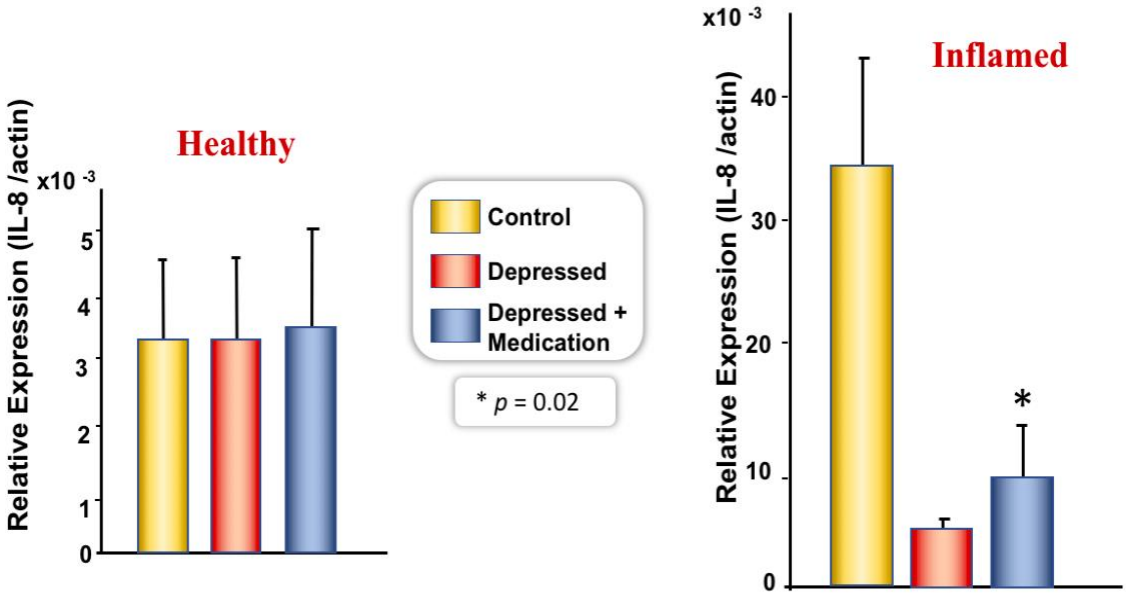
6 Depressed patients have significantly elevated levels of IL1-β in healthy gingival tissue.



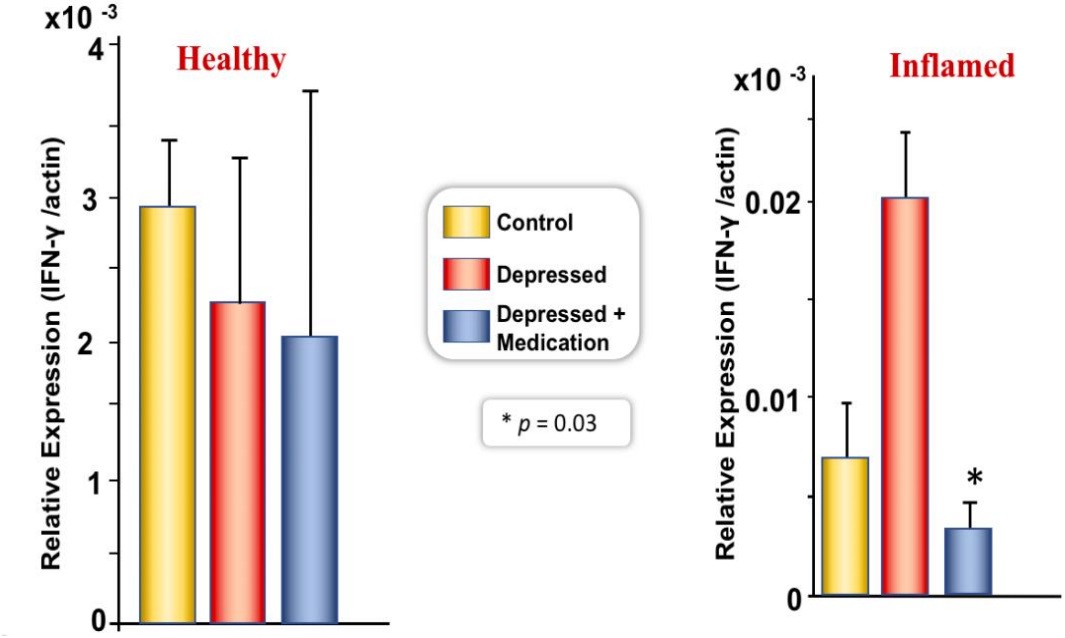
7 Depressed patients have significantly elevated levels of IL-6 in healthy and inflamed gingival tissue.



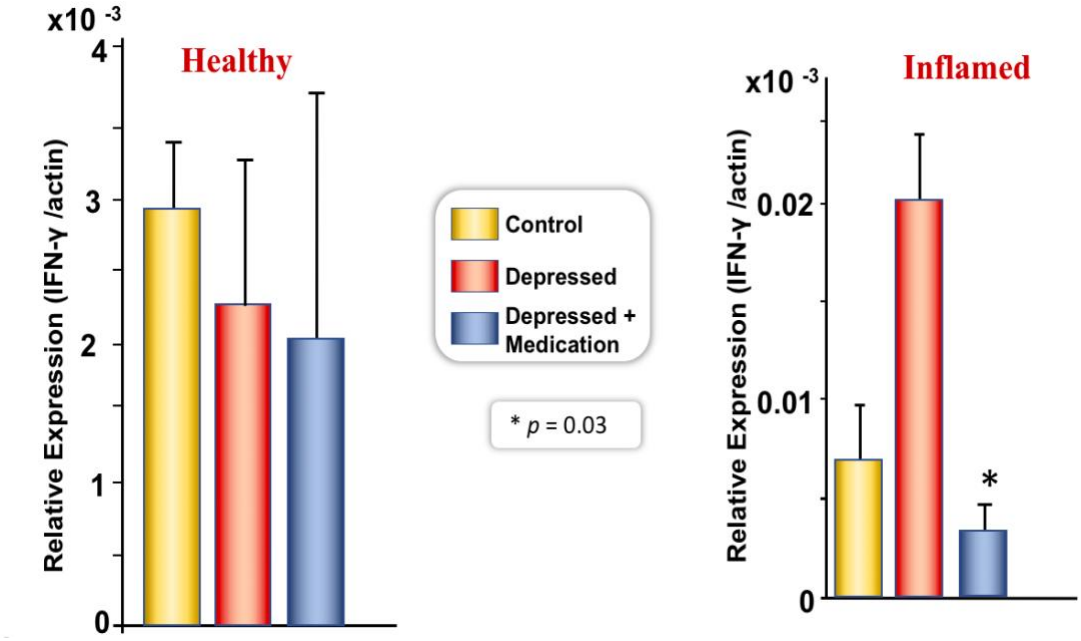
8 Depressed patients have significantly elevated expression of TNF-α.



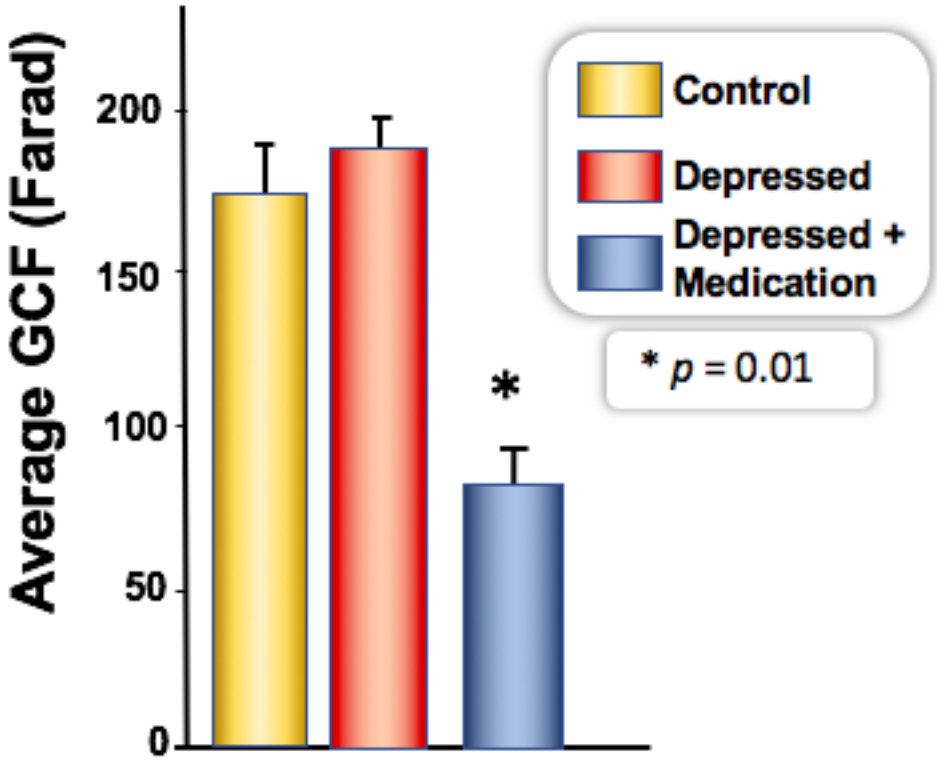
9 IL-8 expression is significantly decreased in inflamed gingival tissue of depressed patients taking medication.



10 Depressed patients taking antidepressant have significantly reduced levels of IFN-γ.



11 IL-10 expression is decreased in depressed patient.



12 Patients taking antidepressants have statistically significant less GCF.

GCF was taken from 3 different sites for each patient and the average of these 3 measurements was used to compare the three different groups.

In the results from the tissue samples is was seen in general that the gene expression of pro-inflammatory cytokines was lower in group 1 when compared to group 2.

Pro inflammatory cytokines

In IL-6, IFN-gamma and IL1- beta we can clearly see this pattern of more gene expression in group 2 than in group 1. For IL-6 group 3 has less expression than group 2 but in IL1 -beta and IFN- gamma there is not a consistent distinction between the gene expression of Group 3 and Group 2. In all circumstances group 1, which is patient taking anti-depressive medication have a clear lower expression of pro-inflammatory markers at a local level.

Anti- inflammatory cytokines

When comparing the expression of anti-inflammatory markers, the for IL-10 there is a clear expression in that group 3 is the one with more expression of these genes. But when comparing group 1 and 2, there is a consistent higher expression of anti-inflammatory gene expression on patients of group 1 compared to patients diagnosed with depression not taking and antidepressant medication in group 2.

There was no significant gene expression when analyzed for COX-2 and IL-2. This corelates with the agarose gel electrophoresis where there is no expression of the gene IL-2.

DISCUSSION

Statistical significance of lower values of IL1-β, IFN-γ and IL-6 gene expression in gingival tissue of patients with antidepressants. Patients taking antidepressant have more expression of IL-10 in the healthy gingival tissue. Statistical significance of lower GCF present in patients with depression already taking antidepressants.

Patients taking antidepressants, group 1, showed that the average of GCF present in pockets deeper than 5 mm is statistically significantly lower than in samples obtained from the patients with depression not taking any medication and patients without any medication. There were significant lower values of IL1-β, IFN-γ and IL-6 gene expression in local tissue in group 1 patients with antidepressants. We could also see a statistically significant higher expression for anti-inflammatory cytokine IL-8.

Even though there is statically significance in some of the genes, it correlates with the literature, since it has been described that the primary indicator of presence of periodontal disease is IL1-β. The novelty is that IL1- β expression is higher in patient with depression and lower in patients taking antidepressants.

This Information may have been a breakthrough to determine whether depression has any significant difference in periodontal status. Further studies are encouraged and must be done to provide more information.

Dentists can evaluate with a simple tool such as the PHQ8 if patients are currently having episodes of depression therefore the dentist can guide the patient to the corresponding

health professional. Patients who are diagnosed with depression and are not taking their antidepressants would have more acceptance towards medication regardless of social stigmas. What this tells us is that patients with undiagnosed depression have a great impact on the periodontal disease progression and in the long term it can progress much faster than a patient that is treating its depression.

Systemic and Local correlation

There is a correlation of systemic gene expression for IL1-β and Il-8 which actually were the genes whose statistical significance was shown present in the oral tissue.

Limitations

Within the limitations there is always human error, in sample collection and lab analysis. This was reduced by having a single doctor collecting all samples from all the subjects who were studied.

Even though there were a large number of patients that were willing to participate there were still conservative people which did not wanted to have their blood drawn, due to fear of needles or other reasons. The time for enrollment and amount patients samples used for the Western blot analysis and RT-PCR was also a short coming and therefore we recommend to continue further studies.

One of the greatest limitations was finding patients that have clinically diagnosed depression and are not taking medication. This is why group 3 was the largest in population size since we kept on trying to find more patients that were diagnosed with depression and not taking antidepressants. This could be addressed next time with help of a mental health clinic so we can have samples of patients that are diagnosed with depression before and after they start with the antidepressant treatment.

Even though the same level of periodontal disease was chosen for all of the groups with a very strict inclusion and exclusion criteria there are still some variables that we as clinicians cannot prevent for example patients daily oral hygiene and compliance.

Western Blot limitations

Western blotting is a technique that separates proteins based on size and it is very technique sensitive. Therefore, the sometimes the band size in the samples analyzed can be different from the predicted. The migration of the protein through the gel is affected by other factors and so the actual band size observed may differ from that predicted. Some of these factors are: post-translational modification (e.g. phosphorylation, glycosylation, which increases the size of the protein), post-translation cleavage (e.g. many proteins are synthesized as pro-proteins and then sliced to give the active form), merged variants, relative charge (depends on the composition of amino acids) and multimers (strong interactions can result in the appearance of higher bands).

Next steps

Other sample analysis

The plaque samples will be used to analyze the levels of red and orange complex bacteria by checkerboard DNA-DNA hybridization. The composition of the microbiomes will be qualitatively assessed using NextGen Sequencing.

GCF samples will be analyzed in the future to measure the cytokine levels (IL-1β, TNF-α, IFN-γ, IL-6, IL-2, IL-8, IL-10) using the LUMINEX assay.

Despite all the short comings, statistically significant data was obtained showing that the pro-inflammatory cytokine IL1- β had an elevated expression in patient with depression and lower in patients taking antidepressants.

CONCLUSION

In this study we have 3 objectives:

1. Evaluate whether use of antidepressants can reduce the expression of inflammatory markers in periodontal disease.

2. Analyze the link between oral and systemic inflammation in patients diagnosed with depression and periodontal disease by comparing cytokine expression systemically (serum) and locally (gingival tissue).

3. Asses volume of gingival crevicular fluid (GCF).

The results demonstrated that there were statistically significant lower values of IL1-β, IFN-γ and IL-6 gene expression in local tissue in group 1 patients with antidepressants.

There is a systemic and local correlation with the gene expression found in the tissue samples and the serum samples. Further studies must be continued to support this breakthrough in the relationship of a systemic factor that affects periodontal disease.

There is statistical significance indicating that there is lower GCF present in patients that have diagnosed depression and are taking their antidepressant treatment adequately.

It has been seen that orally in patients with chronic moderate periodontal disease, there is more pro-inflammatory gene expression of IL-6, IL1-β and IFN-γ in patients that have depression diagnosed and are not treating it.

And if comparing patients with depression both taking medication and not taking medication there was a clear distinction of gene expression of anti-inflammatory markers. The patients taking constantly their antidepressant have more expression of IL-10 at a local level.

Novelty: IL1- β expression is higher in patient with depression and lower in patients taking antidepressants.

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Personal Health Questionnaire Depression Scale (PHQ-8)

Over the **last 2 weeks**, how often have you been bothered by any of the following problems?
*(circle **one** number on each line)*

How often during the past 2 weeks were you bothered by...	Not at all	Several days	More than half the days	Nearly every day
1. Little interest or pleasure in doing things	0	1	2	3
2. Feeling down, depressed, or hopeless	0	1	2	3
3. Trouble falling or staying asleep, or sleeping too much	0	1	2	3
4. Feeling tired or having little energy	0	1	2	3
5. Poor appetite or overeating	0	1	2	3
6. Feeling bad about yourself, or that you are a failure, or have let yourself or your family down	0	1	2	3
7. Trouble concentrating on things, such as reading the newspaper or watching television	0	1	2	3
8. Moving or speaking so slowly that other people could have noticed. Or the opposite – being so fidgety or restless that you have been moving around a lot more than usual	0	1	2	3

Scoring

If two consecutive numbers are circled, score the higher (more distress) number. If the numbers are not consecutive, do not score the item. Score is the sum of the 8 items. If more than 1 item missing, set the value of the scale to missing. A score of 10 or greater is considered major depression, 20 or more is severe major depression.

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Federalwide Assurance # FWA00005960
IORG Registration # IRB00000196 (IRB 01)
IORG Registration # IRB00000726 (IRB 02)

19-Jun-2018

IRB-300000426
Comparing Inflammatory Markers in Patients with and without Depression with Chronic Periodontitis

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The IRB reviewed and approved the Initial Application submitted on 12-Jun-2018 for the above referenced project. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services.

	Full (Institutional Review Board 01 (UAB))
Determination:	Approved
	19-Jun-2018
	One Year
Expiration Date:	18-Jun-2019

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- waiverpartial.clean.180612
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